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Caspase-14 Is a Novel Developmentally Regulated Protease*

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Caspases are a family of cysteine proteases related to interleukin-1 converting enzyme (ICE) and represent the effector arm of the cell death pathway. The zymogen form of all caspases is composed of a prodomain plus large and small catalytic subunits. Herein we report the characterization of a novel caspase, MICE (for mini-ICE), also designated caspase-14, that possesses an unusually short prodomain and is highly expressed in embryonic tissues but absent from all adult tissues examined. In contrast to the other short prodomain caspases (caspase-3, caspase-6, and caspase-7), MICE preferentially associates with large prodomain caspases, including caspase-1, caspase-2, caspase-4, caspase-8, and caspase-10. Also unlike the other short prodomain caspases, MICE was not processed by multiple death stimuli including activation of members of the tumor necrosis factor receptor family and expression of proapoptotic members of the bcl-2 family. Surprisingly, however, overexpression of MICE itself induced apoptosis in MCF7 human breast cancer cells, which was attenuated by traditional caspase inhibitors.

Major advances have been made toward understanding the molecular mechanism of programmed cell death (1). Functioning as central components of the cell death signaling pathway are a rapidly growing family of cysteine proteases that cleave following aspartate residues (caspases)[1] (2, 3). Caspases are normally present as single polypeptide zymogens and contain an N-terminal prodomain and large (p20) and small (p10) catalytic subunits (4–6). The 2-chain active enzyme is obtained following proteolytic processing at internal Asp residues (4–6). As such, caspases are capable of activating each other in a manner analogous to the processing of zymogens observed in the coagulation cascade.

To date, twelve caspases have been identified that can be classified into three subfamilies: caspase-1 (interleukin-1 converting enzyme), caspase-4 (ICErelII, TX, ICH2), caspase-5 (ICErelIII, TY), caspase-11 (Ich-3), and caspase-12 belong to the caspase-1 subfamily; caspase-2 (Ich-1) is the sole member of the caspase-2 subfamily; caspase-8 (FLICE, MACH, Mch5), caspase-9 (ICE-LAP6, Mch6), caspase-10 (FLICE2, Mch4), caspase-3 (Yama, CPP32, apopain), caspase-7 (ICE-LAP3, Mch3, CMH-1), and caspase-6 (Mch2) belong to the caspase-3 subfamily (2, 3, 7). An alternate classification is based on the size of the prodomain because large prodomain caspases function as upstream signal transducers, whereas short prodomain caspases function as downstream amplifiers that cleave death substrates (8). It is not entirely clear how large prodomain caspases are activated; however, recent studies suggest that their binding to receptor-associated adaptor molecules results in their approximation and activation by autoprocessing (8–12).

Three short prodomain caspases exist in the caspase-3 subfamily, whereas none have been found in the other two subfamilies (2, 3). Here we report a novel developmentally regulated short prodomain caspase designated MICE or caspase-14 that is a member of the caspase-1 subfamily and possesses unique biochemical properties.

MATERIALS AND METHODS

Cell Lines and Expression Vectors—Human embryonic kidney 293 and 293-EBNA cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Human breast carcinoma MCF7 cells were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Expression constructs of tumor necrosis factor receptor family members were in pFLAG-CMV-1 (Kodak). Bax, Bak, and Bik expression constructs were generously provided by G. Chinnadurai, IAP1 and IAP2 by D. V. Goeddel, and Hrk by G. Nunez. All other expression constructs were made in pcDNA3 (Invitrogen). Epitope tags were placed at the C termini unless otherwise indicated.

Cloning of Caspase-14 (MICE)—cDNA sequences corresponding to the partial open reading frame of caspase-14 were identified as expressed sequence tags (EST) (GenBank™ accession numbers AA103647 and AA167930) homologous to caspase family members. Both clones were sequenced using plasmid DNA template by the dideoxy chain termination method employing modified T7 DNA polymerase (Sequenase, United States Biochemical). The AUI epitope-tagged version of MICE was obtained by polymerase chain reaction using custom synthesized primers.

Northern Blotting—Mouse adult multiple tissue and embryo tissue poly $(A)^+$ RNA blots were obtained from CLONTECH and processed according to manufacturer instructions. A ^{32}P-labeled cDNA corresponding to MICE amino acid residues 44–152 was used as probe.

Transfection, Coimmunoprecipitation, and Western Analysis—Transient transfections of 293 cells were performed as described previously (13). Cells were harvested 20–30 h following transfection and either immunoprecipitated and immunoblotted or directly immunoblotted with the indicated antibodies.

Cell-Death Assay—293 EBNA cells and MCF7 cells were transiently transfected with 0.1 and 0.25 μg of the reporter plasmid pCMV β-galactosidase, respectively, plus 0.5–1.0 μg of test plasmids in the presence or absence of 2.0 μg of inhibitory plasmids. 24–30 h following transfection, cells were fixed with 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside. Percentage of apoptotic cells was determined by calculating the fraction of membrane-blebbed blue cells as a function of total blue cells. All assays were

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[1] The abbreviations used are: caspase, cysteine aspartate specific protease; ICE, interleukine-1 converting enzyme; MICE, mini-ICE; I-FLICE, inhibitor of Fas-associated death domain protein (FADD)-like ICE; TNFR1, tumor necrosis factor receptor 1; IAP, inhibitor of apoptosis; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

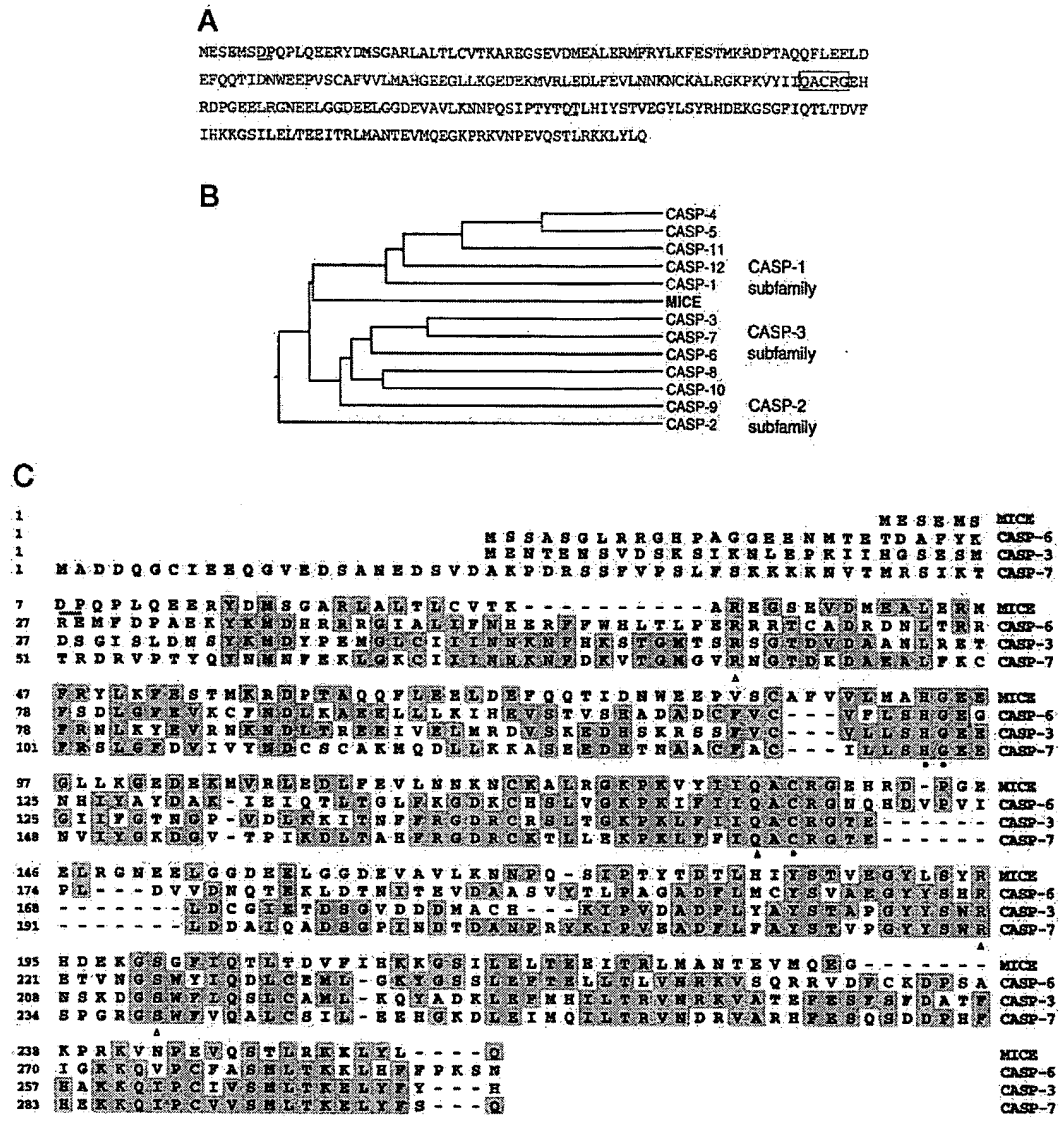

FIG. 1. **Sequence analysis of MICE.** *A*, deduced amino acid sequence of MICE. The conserved pentapeptide QACRG is *boxed*, and the putative cleavage site between prodomain and p20 and that between p20 and p10 are *underlined*. *B*, phylogenetic analysis of caspase family. *C*, sequence alignment of MICE and three known short prodomain caspases. *Solid circles* indicate residues involved in catalysis, and *triangles* identify residues that form the binding pocket for the carboxylate side chain of P1 Asp. The putative cleavage sites between prodomain and p20 are *underlined*. *CASP-*, caspase.

evaluated in duplicate, and the mean and standard deviation was calculated.

Mice Expression and Purification—Recombinant MICE was expressed in *Escherichia coli* strain Bl21 (DE3) plysS following induction for 4 h at 37 °C with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation, resuspended in 100 mM Tris-HCl, 100 mM NaCl, pH 8, and lysed by freeze-thaw cycles followed by sonication. The supernatant was recovered by centrifugation and applied directly to immobilized Ni-nitrilotriacetic acid for purification utilizing the engineered N-terminal His tag. The protein was eluted with a 0–200 mM imidazole gradient, and the recovered MICE was contaminated with an equal amount of *E. coli* histidine-rich protein. Final purification of MICE was achieved by ion exchange utilizing a gradient of 0–500 mM NaCl in 20 mM Tris-HCl following adsorption to DEAE-Sepharose. Approximately 1 mg of MICE was obtained from 3 liters of *E. coli*, and the final concentration was 0.3 mg/ml.

Mice Activity Assay—Purified MICE (10 μl) was added to 40 μl of caspase assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2) in the absence (control) or presence of the following caspase inhibitors: 2 μM Z-VAD-FMK, or 5 μM CrmA(REF) or 0.3 μM p35, followed by incubation at 37 °C for 30 min to allow for complex formation. Residual activity was assayed by adding 50 μl of a 0.2 mM solution of the caspase substrate Ac-DEVD-AFC, and measurement of released AFC at 37 °C using a Perkin-Elmer LS50 spectrofluorimeter with excitation at 400 nm and emission at 505 nm.

RESULTS AND DISCUSSION

MICE Is a Short Prodomain Caspase of Caspase-1 Subfamily—Analysis of the full-length cDNA sequence of MICE revealed a 774-base pair open reading frame that encoded a novel protein of 257 amino acids with a predicted molecular mass of 29.5 kDa (Fig. 1*A*). Comparison of this protein with all known caspases revealed that it had a unusually short prodomain of only six amino acids (Fig. 1, *A* and *C*). Given this, the molecule was termed MICE (for mini-ICE). The caspase designation for it is caspase-14.

Phylogenetic analysis revealed MICE to be most related to caspase-1 subfamily members, and it is therefore the first short prodomain caspase to be part of the caspase-1 subfamily (Fig. 1*B*). Overall, MICE displayed 21.4, 19.5, and 20.2% identity to

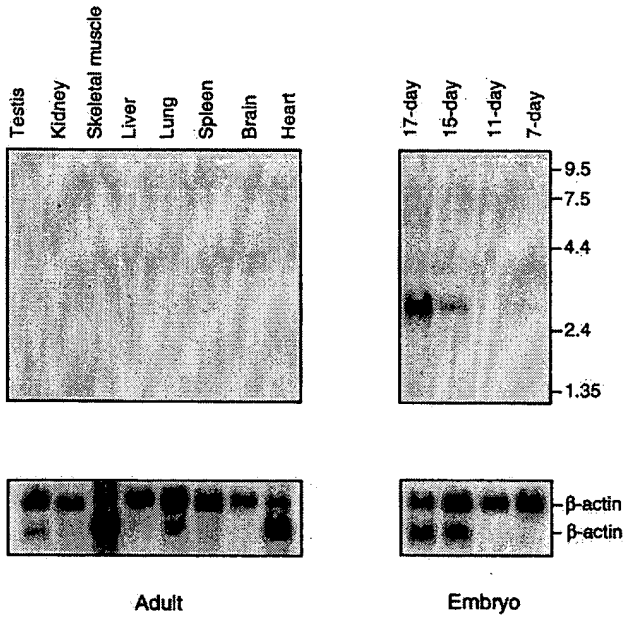

FIG. 2. **Tissue distribution of MICE.** Murine adult multiple and embryo tissue poly(A)$^+$ Northern blots were probed with ^{32}P-labeled MICE cDNA.

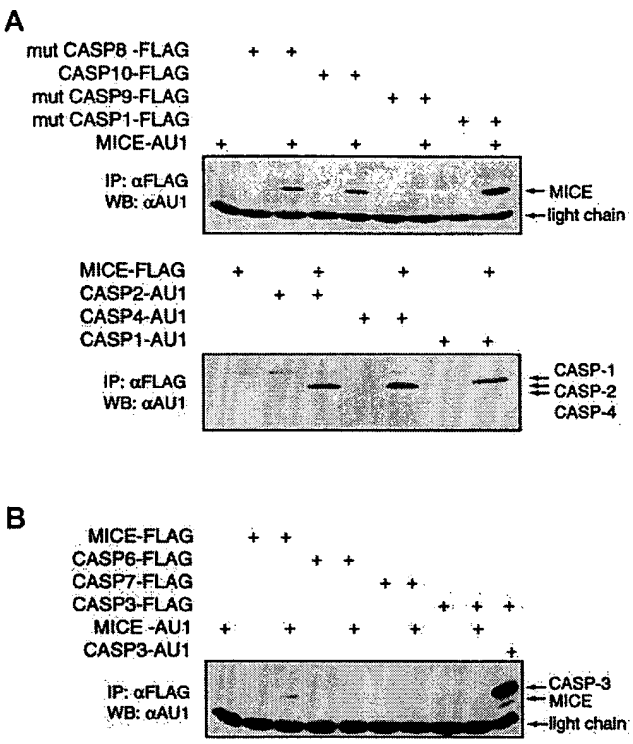

FIG. 3. **MICE preferentially associates with some large prodomain caspases.** 293 cells were co-transfected with the expression constructs encoding epitope-tagged MICE and other caspases. 30 h following transfection, cells were harvested, lysed, and analyzed with the indicated antibodies. The expression of each plasmid was confirmed by either reprobing the blots or directly blotting the cell lysate. *mut*, mutant; *CASP-*, caspase.

the known short prodomain caspases 3, 6, and 7, respectively (Fig. 1*C*). The QACRG pentapeptide motif present in most caspases is also conserved in this novel caspase. In addition, based on the x-ray crystal structure of caspase-1 and caspase-3, amino acid residues involved in catalysis are conserved in MICE as are residues that form a binding pocket for the carboxylate side chain of the P1 aspartic acid (Fig. 1*C*) (4–6). This is in keeping with MICE being a functional caspase.

Tissue Distribution of MICE—Mouse adult and embryonic tissue poly(A)$^+$ RNA blots were probed with a ^{32}P-labeled cDNA corresponding to the large catalytic subunit of MICE. A single transcript of 2.8 kilobases was observed (Fig. 2). Unlike almost all known caspases that are expressed in both adult and embryonic tissues (7, 10, 11, 14–18), MICE was highly expressed in certain stages of embryonic development but was undetectable in all adult tissues examined, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. Interestingly, the expression level of MICE appeared to increase during the later stages of development (the attenuated expression in day 11 may be because of lower loading of mRNA).

MICE Preferentially Associates with Certain Large Prodomain Caspases—Because small prodomain caspases function downstream of large prodomain caspases, we asked if MICE, being a short prodomain caspase, bound any of the putative upstream large prodomain caspases. Surprisingly, MICE associated with most large prodomain caspases, including caspase-1, -2, -4, -8, and -10 (Fig. 3*A*). The other short prodomain caspases including caspase-3, -6, and -7 associated only with caspase-10 (data not shown). MICE did not bind the other short prodomain caspases and displayed only weak self-association in contrast to caspase-3 and other caspases that strongly self-associated (Fig. 3*B*, and unpublished data). The preferential dimerization with large prodomain caspases and weak self-association suggest that MICE may function through heterodimerization.

MICE Is Not Processed in Multiple Death Signaling Pathways—Previous studies have shown that initially long prodomain and then short pro-domain caspases are processed following activation of death receptors including TNFR1 and CD95 death receptors (19–24). To further characterize MICE, we asked if it was processed on activation of these receptors. 293 cells were transiently transfected with expression constructs encoding death signaling receptors and MICE, or the three known short prodomain caspases: caspase-3, -7, and -6. Interestingly, all three known short prodomain caspases were processed upon coexpression with the death signaling receptors (Fig. 4*A*). MICE, however, was not processed, suggesting that it is not involved in the death pathway engaged by these proapoptotic receptors (Fig. 4*A*).

Bax, Bak, Bik, Bad, Bid, and Hrk are proapoptotic members of the bcl-2 family (23, 25). 293 cells were transiently transfected with expression constructs encoding short prodomain caspases and proapoptotic bcl-2 family members. In keeping with the prior results, all three known short prodomain caspases were processed on co-expression, but MICE was not processed (Fig. 4*B*).

Because MICE was not processed on activation of a number of distinct physiologically relevant death pathways, we asked if it could serve as a substrate for known caspases. Expression constructs encoding MICE and known caspases were coexpressed in the presence or absence of the death signaling receptor TNFR1. Consistent with previous results, no processing of MICE was observed despite the additional death signal from TNFR1 (Fig. 4*C*). MICE was also not processed by caspase-1 or -4, both members of the caspase-1 subfamily (data not shown). The failure of processing of MICE suggests that it likely functions in a very specific pathway that remains to be defined.

MICE-induced Apoptosis Is Attenuated by Inhibitors of Apoptosis—To determine whether MICE plays a role in cell death, 293 EBNA and MCF7 cells were transfected with expression plasmids encoding wild-type MICE, a mutant version of MICE

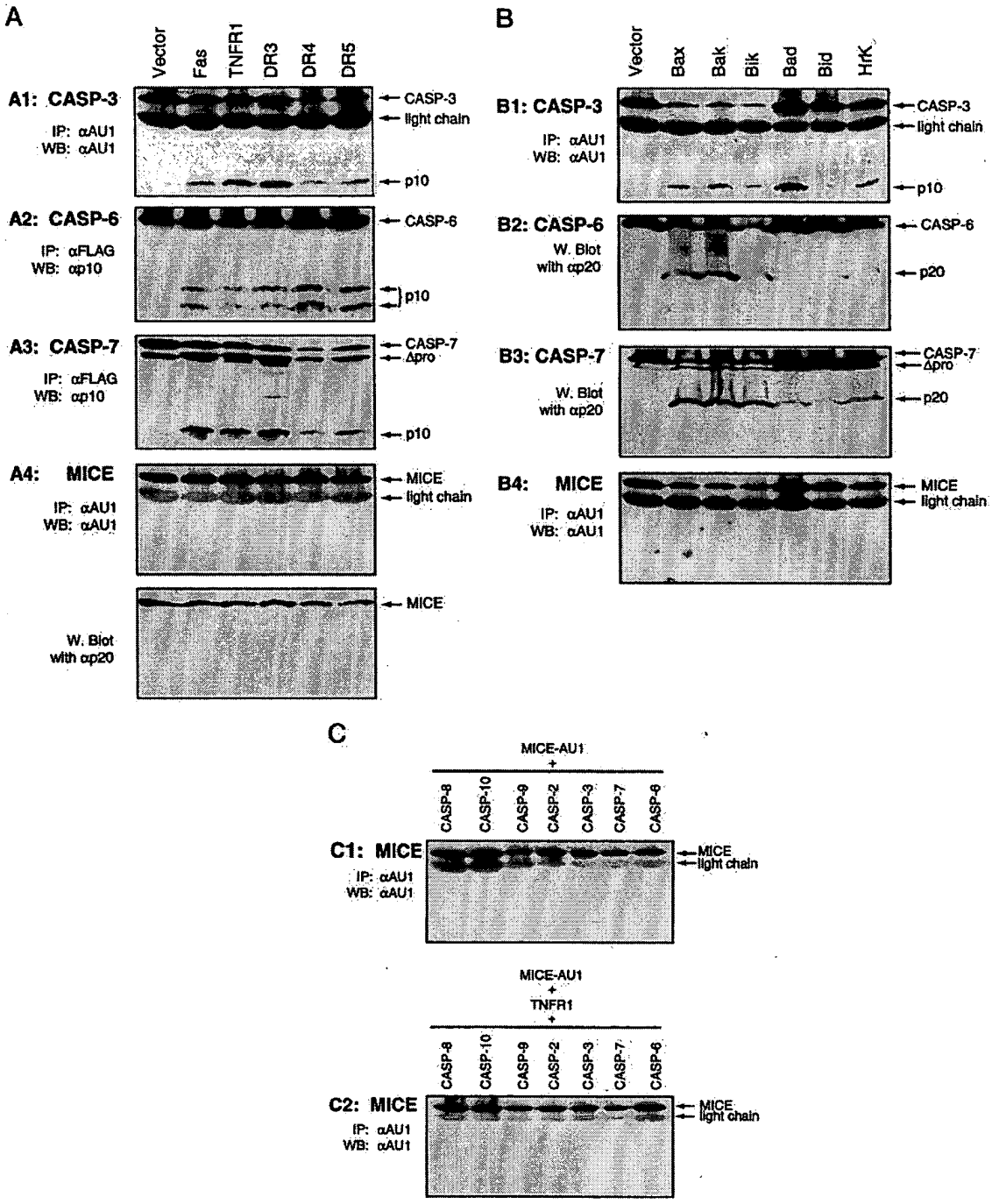

FIG. 4. **MICE is not processed on activation of known death signaling pathways.** 293 cells were co-transfected with expression constructs encoding TNFR family members and either C-terminal-tagged caspase-3-AU1, caspase-6-FLAG, caspase-7-FLAG, or MICE-AU1 (*A*). MICE was also co-expressed with the proapoptotic members of bcl-2 family (*B*) or caspases in the absence or presence of TNFR1 (*C*). 20–24 h following transfection, cells were harvested and either immunoprecipitated (*IP*) and immunoblotted or directly immunoblotted with the indicated antibodies. *WB*, Western blot; *CASP-*, caspase; *IP*, immunoprecipitated.

in which the presumed catalytic cysteine was altered to an alanine (QACRG to QAARG mutant) and caspase-8 as a positive control that has previously been shown to potently induce apoptosis in both cell lines (9, 10). Like the three other known short prodomain caspases, MICE had little effect on 293 EBNA cells (Fig. 5*A*, and unpublished data). However, it induced apoptosis in MCF7 cells (Fig. 5*A*). As expected, catalytically inactive MICE displayed substantially less death-inducing activity. More importantly, MICE-induced apoptosis in MCF7 was inhibited by the baculoviral-encoded inhibitors of apoptosis 1 and 2 (IAP1 and IAP2) and the broad spectrum baculoviral caspase inhibitor p35, but not by CrmA, MC159, or I-FLICE (Fig. 5*B*). CrmA is a cowpox serpin that inhibits caspase-1 and -8 activity, whereas MC159 is a death effector domain-containing decoy molecule encoded by molluscum contagiosum virus (26–27). I-FLICE is a naturally occurring catalytically inert dominant-negative caspase (28). These inhibitors function at the apex of the apoptotic cascade by disrupting assembly of

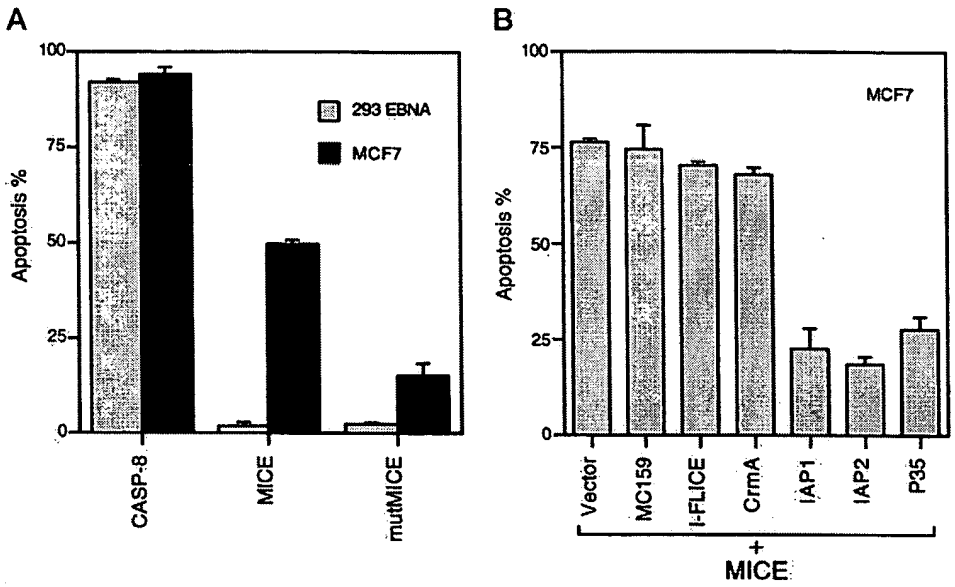

FIG. 5. **MICE-induced apoptosis is attenuated by the inhibitors of apoptosis.** *A*, 293 and MCF7 cells were co-transfected with a reporter gene (β-galactosidase) and expression constructs encoding caspase-8 (*CASP-8*), MICE, and a mutant version of MICE (*mutMICE*). *B*, MCF7 cells were co-transfected with the reporter gene and MICE expression construct in the absence or presence of the inhibitor plasmids. The cells were fixed and stained as described under "Materials and Methods." Expression of all transfected plasmids was verified by immunoblotting.

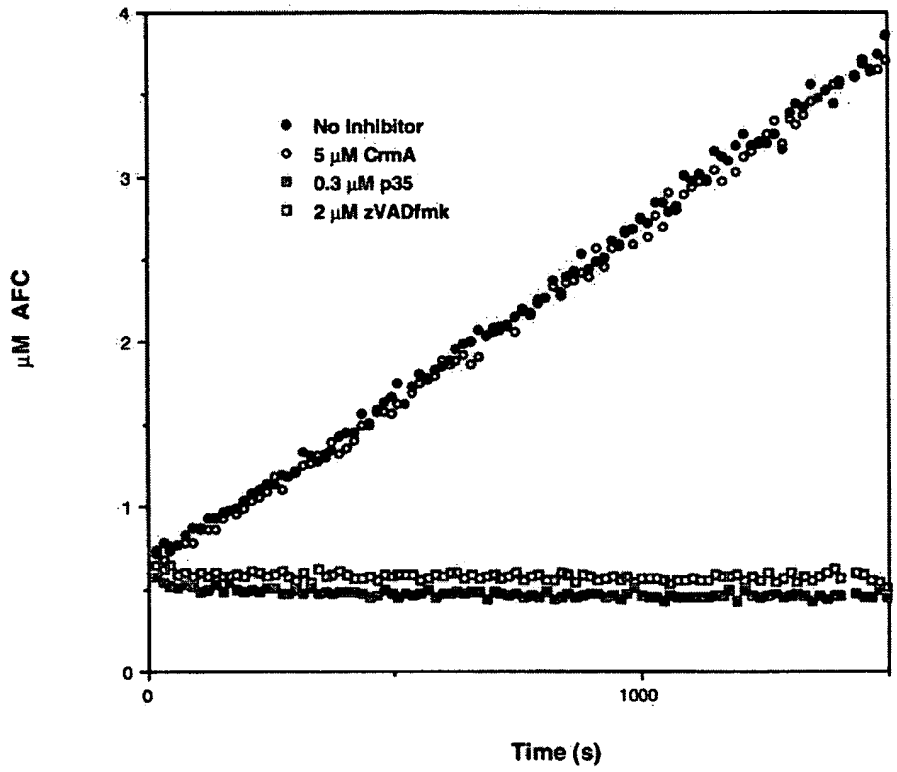

FIG. 6. **Characterization of MICE caspase activity.** The ability of recombinant MICE to cleave the fluorometric caspase substrate Ac-DEVD-AFc is shown as a function of the inhibitor present at time of initiation of assay.

receptor signaling complexes and/or inhibiting the initiating caspase (27–29). Because they had no effect on cell death induced by MICE, it is possible that MICE functions as a downstream signal transducer of cell death. However, it should be emphasized that the results from these overexpression studies should not be construed to imply a definitive role for caspase-14 in apoptosis. This will have to await more definitive studies including the generation of a mouse that is homozygous null for the gene in question.

MICE Possesses Caspase Activity—Recombinant MICE prepared by overexpression in *E. coli* possessed intrinsic caspase activity that was inhibitable by the broad spectrum caspase inhibitors zVADfmk and p35 but not by CrmA (Fig. 6).

In summary, the failure of MICE to undergo processing in multiple known death pathways and its ability to physically interact with large prodomain caspases and induce cell death suggests that MICE likely functions as a downstream active caspase in an as yet unidentified signaling pathway.

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